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Humoral and Cellular Immune Responses to *Yersinia pestis* Infection in Long-Term Recovered Plague Patients

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Plague is one of the most dangerous diseases and is caused by *Yersinia pestis*. Effective vaccine development requires understanding of immune protective mechanisms against the bacterium in humans. In this study, the humoral and memory cellular immune responses in plague patients ($n = 65$) recovered from *Y. pestis* infection during the past 16 years were investigated using a protein microarray and an enzyme-linked immunosorbent spot assay (ELISpot). The seroprevalence to the F1 antigen in all recovered patients is 78.5%. In patients infected more than a decade ago, the antibody-positive rate still remains 69.5%. There is no difference in the antibody presence between gender, age, and infected years, but it seems to be associated with the F1 antibody titers during infection ($r = 0.821$; $P < 0.05$). Except F1 antibody, the antibodies against LcrV and YopD were detected in most of the patients, suggesting they could be the potential diagnostic markers for detecting the infection of F1-negative strains. Regarding cellular immunity, the cell number producing gamma interferon (IFN-γ), stimulated by F1 and LcrV, respectively, *in vitro* to the peripheral blood mononuclear cells of 7 plague patients and 4 negative controls, showed no significant difference, indicating F1 and LcrV are not dominant T cell antigens against plague for a longer time in humans. Our findings have direct implications for the future design and development of effective vaccines against *Y. pestis* infection and the development of new target-based diagnostics.

Plague is a deadly infectious disease caused by *Yersinia pestis* and there are 1,000 to 5,000 human plague cases reported each year worldwide (20). Although the fatality rate of infected persons can decrease dramatically if they are treated by effective antibiotics on time, the existence of antibiotic-resistant virulent *Y. pestis* strains indicates that an effective vaccine against both bubonic and pneumonic plagues is urgently needed, and the potential misuse for biological warfare or bioterrorism also strengthens this need (5, 8).

Three types of vaccines, namely, killed whole-cell (KWC) vaccines, live attenuated vaccines (EV76), and recombinant subunit vaccines, have been developed against plague. Although KWC and EV76 vaccines provide protection against plague in animal models, both have side effects and need repeated immunizations for developing immunity in humans (19, 29, 30). They are no longer used in humans in the Western world. EV76 is still the vaccine of choice for humans in China. Subunit vaccines based on the capsular protein F1 and one of the type III secretion system proteins, LcrV, have been the focus of recent efforts (1, 9, 24, 28, 32). This subunit vaccine has been shown to protect mice against respiratory infection by *Y. pestis* and has been reported for entry into a phase II study (9, 34). However, it failed to adequately protect African green monkeys from pneumonic plague (26). Moreover, the F1 mutant and the LcrV variant strains can possibly circumvent the effectiveness of this subunit vaccine (36). This highlights the need to identify novel and effective vaccines that can address all forms of plague.

Understanding of the antimicrobial immune responses of the host will enable the discovery of more effective vaccines. The immune mechanism against *Y. pestis* is extremely complex and involves a combination of humoral and cellular factors (14). Studies have focused on the antibody-based humoral immunity, and the majority of these studies employed animal plague models, which cannot reflect the real immune protective mechanisms of humans.

In contrast to the approximately 6 to 12 months of protection in EV76-immunized people (6), individuals who survived the plague infection could establish the protective responses. They are considered to have acquired immunity against subsequent reinfection of *Y. pestis*. The immune responses to *Y. pestis* of recovered patients and the persistence of *Y. pestis*-induced immunity after infection will provide the most important data that can facilitate the development of effective vaccine.

Although previous studies confirmed that the F1 antibody could persist for 1 to 4 years in humans (18, 27), there is no report on longer persistence of the F1 antibody and the existence of the antibodies against proteins other than F1 in patients in the long term. In the present study, the serum samples from 65 plague patients who were in recovery for more than 10 years were collected and screened by protein microarray to investigate antibody profile. Meanwhile, the specific memory T cell responses to F1 and LcrV proteins in the recovered patients were also analyzed.

Received 3 November 2011 **Returned for modification** 28 November 2011
Accepted 9 December 2011
Published ahead of print 21 December 2011
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doi:10.1128/CVI.05559-11

TABLE 1 Information on recovered plague patients

Patient no.	Gender	Age at infection (years)	Diagnosis year	Residing county	Result of IHA at infection (titer)	Results of UPT in 2006 (T/C)
1	Female	13	1990	Lincang	1:2,560	19.111
2	Female	45	1990	Lincang	1:20	0.054
3	Male	71	1990	Lincang	1:160	5.073
4	Male	7	1990	Lincang	1:20	0.196
5	Female	36	1990	Lincang	1:320	6.479
6	Female	20	1990	Lincang	1:640	13.723
7	Male	55	1990	Lincang	1:20	0.294
8	Male	16	1990	Lincang	1:160	0.982
9	Female	8	1990	Lincang	1:640	6.9
10	Male	20	1990	Lincang	1:320	5.652
11	Female	4	1990	Lincang	1:160	0.638
12	Male	7	1990	Lincang	1:80	0.108
13	Male	19	1990	Lincang	1:80	0.375
14	Female	24	1992	Yuxi	1:40	0.067
15	Female	44	1992	Yuxi	1:20	0.079
16	Female	42	1992	Yuxi	1:40	0.027
17	Female	67	1996	Honghe	1:1,280	1.833
18	Female	8	1996	Honghe	1:80	0.073
19	Male	28	1996	Honghe	1:80	0.428
20	Male	9	1996	Honghe	1:320	1.453
21	Female	49	1996	Honghe	1:20	0.073
22	Male	53	1996	Honghe	1:20	0.094
23	Male	7	1996	Honghe	1:40	0.204
24	Female	19	1997	Kunming	1:160	0.321
25	Male	3	1997	Kunming	1:320	3.183
26	Male	12	1997	Kunming	1:160	3.115
27	Male	45	1997	Kunming	1:640	1.734
28	Male	8	1997	Kunming	1:80	1.341
29	Female	41	1997	Kunming	1:160	3.488
30	Male	25	1997	Kunming	1:80	0.158
31	Male	49	1997	Kunming	1:160	2.531
32	Male	43	1997	Kunming	1:160	1.258
33	Female	27	2000	Honghe	1:40	0.294
34	Male	47	2000	Honghe	1:80	0.055
35	Female	46	2000	Honghe	1:80	0.055
36	Male	50	2000	Honghe	1:320	0.131
37	Male	44	2000	Honghe	1:160	0.281
38	Female	32	2000	Honghe	1:40	0.17
39	Male	24	2000	Honghe	1:20	0.035
40	Female	29	2000	Honghe	1:20	0.022
41	Female	68	2002	Honghe	1:160	1.548
42	Female	27	2002	Honghe	1:640	3.13
43	Female	47	2002	Honghe	1:80	0.157
44	Female	28	2002	Honghe	1:320	3.68
45	Male	38	2002	Honghe	1:20	0.077
46	Female	13	2002	Honghe	1:40	4.866
47	Male	39	2002	Honghe	1:20	1.573
48	Female	57	2002	Honghe	1:80	0.5
49	Female	44	2002	Honghe	1:80	0.22
50	Male	34	2002	Honghe	1:80	0.284
51	Female	35	2002	Honghe	1:20	0.179
52	Female	41	2002	Honghe	1:80	0.567
53	Female	31	2002	Honghe	1:80	0.395
54	Male	28	2002	Honghe	1:20	0.099
55	Male	53	2002	Honghe	1:20	0.095
56	Male	32	2002	Honghe	1:40	0.142
57	Male	41	2003	Dehong	1:1,280	13.62

(Continued on following page)

TABLE 1 (Continued)

Patient no.	Gender	Age at infection (years)	Diagnosis year	Residing county	Result of IHA at infection (titer)	Results of UPT in 2006 (T/C)
58	Female	14	2003	Dehong	1:40	2.381
59	Female	53	2003	Dehong	1:320	1.546
60	Female	8	2003	Dehong	1:640	3.025
61	Male	15	2003	Dehong	1:40	0.207
62	Male	33	2003	Dehong	1:1,280	19.411
63	Male	54	2005	Yulong	1:80	1.126
64	Male	41	2005	Yulong	1:80	1.444
65	Male	27	2005	Yulong	1:20	0.334

MATERIALS AND METHODS

Serum samples from recovered plague patients. Sixty-five patients infected by *Y. pestis* from the *Rattus flavipectus* plague foci in the Yunnan-Guangxi-Fujian provinces of China were recruited for blood sampling in May 2006. They were diagnosed to have recovered from *Y. pestis* infection according to the clinical criteria and serodiagnosis against F1 antigen with the indirect hemagglutination assay (IHA) between 1990 and 2005. All patients stated that they did not experience reinfections and have not received immunization against *Yersinia pestis* after primary infection. The details in regard to gender, age, infection time, and the F1 antibody titer at the time of infection are provided in Table 1. The sera from the subjects were collected and stored at −20°C for further use. Forty-eight serum samples were collected from persons with no plague history in the areas of endemicity. Forty-three serum samples were collected from persons in counties of nonendemicity and were used as negative controls.

Detection of antibodies against F1 by up-converting phosphor technology-based lateral flow (UPT-LF) and enzyme-linked immunosorbent assay (ELISA). All collected sera were screened for the antibody against F1 by F1 antigen-based UPT-LF, which is a quantitative assay developed recently for detecting microorganisms and antibodies (10, 17, 25). For developing double-antigen sandwich LF strips to detect F1 antibody, F1 antigen (1 mg/ml, 1 μl/cm) and their corresponding antibodies (1 mg/ml, 1 μl/cm) were dispensed on the nitrocellulose membrane as the test line (T) and control line (C), respectively. Up-converting phosphor (UCP)-F1 antigen conjugate (1 mg/ml, 30 μl/cm) was fixed in the glass fiber as the conjugate pad. The result of the UPT-LF strip was analyzed by UPT biosensor. The areas of the peaks corresponding to the test and control lines were referred to as T and C, respectively, and the ratio of T/C is the result of measurement. Samples with a T/C ratio higher than the cutoff threshold (mean plus 3 standard deviations [SD]) were regarded as positive and vice versa (10). To confirm the results of UPT, the F1 antibody titer in the recovered patients was tested using ELISA, which was validated by the Institute Pasteur de Madagascar in 1995 (27). The sera of healthy blood donors were used to define a cutoff value for determining positive or negative results.

Antibody screening by protein microarray. The protein microarray included 218 outer membrane proteins, surface-exposed or secreted proteins, and known or putative virulence factors and the genes located in several genomic islands that were likely acquired in the evolution of *Y. pestis*. These were constructed according to previous reports (12).

According to the results of UPT, 2 to 4 sera of patients who were infected in the same year and had similar UPT results of F1 antigen were pooled for antibody profiling against the proteins on the microarray. The profiling process and data analysis were performed based on the procedures of earlier studies (12, 13, 15). Six serum samples of healthy people that were negative for the F1 antibody were used as negative controls.

F1- and LcrV-specific gamma interferon (IFN-γ) production in recovered patients by enzyme-linked immunosorbent spot (ELISpot) assay. Recombinant F1 and LcrV proteins were expressed in *Escherichia coli* and purified as previously described (16). ELISpot assays were performed using a commercially available kit (BD Pharmingen) according to the manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) of 7 patients (patient no. 36, 37, 38, 41, 45, 47, and 51 in Table 1) who recovered from the plague 4 to 6 years ago were isolated from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. The number of cells was adjusted as required prior to stimulation. Purified recombinant proteins F1 and LcrV (10 μg/ml), phytohemagglutinin (5 μg/ml), or complete medium (RPMI 1640 medium) were added to the cells in triplicate. The resulting spots were counted using a cytotoxic T lymphocyte immunospot analyzer. The final numbers of IFN-γ-secreting cells stimulated by F1 and LcrV proteins were determined from the results of triplicate wells as the mean numbers of cytokine spots per million cells from patients. The number of background spots (those for IFN-γ-producing T cells from complete medium stimulated *in vitro*) was subtracted. PBMCs from 4 healthy donors in Beijing, China, were used as negative controls.

Statistical analysis. The association between prevalence of the F1 antibody-positive rate and gender, age, and years of infection was examined by the x^2 test. Pearson correlation coefficients (r) were calculated to determine correlations between the anti-F1 titers of patients at infection and the remaining amount of the F1 antibody after several years to more than a decade postinfection. Differences in the numbers of IFN-γ-producing cells between recovered plague patients and healthy control donors were compared using Student's t test. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Antibodies against F1 in the recovered plague patients. The study subjects consist of 65 (34 male and 31 female) plague patients who were recovered from bubonic plague between 1990 and 2005. All serum samples were screened with UPT-LF to detect the amount of antibody against the F1 antigen, an antigen for the serodiagnosis of plague in human patients and infected animals. Fifty-one of 65 recovered patients (78.5%) were positive, suggesting that the F1 antibody in serum could persist from several years to more than a decade after infection. The prevalence of the F1 antibody was 88% among patients infected within 5 years, and its positive ratio decreased to 69.5% in patients infected for more than a decade (Table 2). Only 1 of 13 patients infected in 1990 was negative for the F1 antibody in serum, and the remaining 12 patients remained positive for F1 antibody in sera (Table 1). However, all of the 3 serum samples from the patients infected in 1992 were negative for the F1 antibody (3/3, 100%). This indicates that the antibody persistence in recovered patients may be determined not only by the time after infection. It might be mainly influenced by individual differences. Five persons in counties where plague is endemic and one person in a county where plague is nonendemic were also positive for the F1 antibody in sera with considerably lower antibody quantity than those in the recovered patients (see Tables 2 and 4). Although several studies proved that anti-F1 an-

TABLE 2 Detection of F1 antibody in different groups by UPT and ELISA

Method	No. positive/tested (positive rate %)				No. of people from areas of endemicity	No. of people from areas of nonendemicity
	Infection time					
	≤5 years	5–10 years	≥10 years	Total		
UPT	22/25 (88)	13/17 (76.5)	16/23 (69.5)	51/65 (78.5)	5/48 (10.4)	1/43 (2.3)
ELISA	22/25 (88)	13/17 (76.5)	16/23 (69.5)	51/65 (78.5)	6/48 (12.5)	1/43 (2.3)

tibodies could persist for several years in humans (18, 27), our study was the first to determine the persistence of the F1 antibody in patients for more than a decade postinfection.

The factors in relation to persistent time of the F1 antibody in recovered patients. Previous studies have shown that plague antibodies are more prevalent in males in populations exposed to infection, and the difference in relation to age was also reported (4, 7). However, the factors in relation to the time of persistence and the amount of the F1 antibody in humans have not been investigated before. Among the 65 recovered patients, the F1 antibody was detected in 23 of the 31 females (74.2%) and 28 of the 34 males (82.4%). The seroprevalence rate is not significantly different in terms of gender ($P = 0.424$), suggesting that persistence of the F1 antibody in recovered patients was not related to sex. It is not related to time periods after infection (88%, 76.5%, and 69.5% in ≤5 years, 5 to 10 years, and ≥10 years postinfection, respectively; $P = 0.292$). Although the positive rate in patients who were recovered for ≥10 years was slightly lower than those who were for recovered ≤5 years, the seroprevalence in patients who were recovered for 16 years was 92.3% (12/13). Because the safety and immunogenicity data of vaccine in people younger than 18 were not available, the recovered patients were divided into <18- and ≥18-year-old groups to study the relationship between age and the positivity of the F1 antibody. The seropositivity of the F1 antibody showed no difference between the two groups (15/16 versus 36/49; $P = 0.087$) (Table 3). This indicates that the duration of the antibody might not be influenced by age of infection.

The UPT values of recovered patients correlated well with the IHA titers of the corresponding patient at infection ($r = 0.821$; $P < 0.05$) (Fig. 1). Patients with higher F1 antibody titers at infection seemed to retain higher levels of the F1 antibody value in the body after recovery. Serum antibody titers against F1 antigen are correlated with the degree of protection against *Y. pestis* infection in experimental animals (35). The long-term high level of the F1 antibody in the serum of the recovered patients may be one of the reasons that they are protected from plague. Although the F1 antibody in 68% (36/50) of EV76-immunized persons was also detected by UPT-LF in 2006, which is approximately 15 years after immunization (Table 4), the F1 antibody value was significantly lower than those in the recovered patients ($P < 0.01$).

EV76 is a live attenuated vaccine lacking *pgm* locus which has been widely used in China and in the former Soviet Union in the past (29). Unlike the majority of the recovered patients who could acquire long-term protection, the protective duration of EV76-immunized people is approximately 6 to 12 months (6). The lower F1 antibody level in EV76-immunized people than that in the recovered patients may explain the poor protection of EV76 against plague (Table 4). Although the EV76 strain can live in humans and stimulate immune responses, the lack of *pgm* locus could influence its survival ability in humans, limiting its replication and dissemination and leading to insufficient contact with immune cells.

Antibody profiling in patients infected at different years. Except for F1 protein, the antibody to many other proteins, such as LcrV, YopH, YopE, and YopD, can also be detected in the acute and convalescent-phase sera of plague patients (2, 3). To study the time of persistence of antibodies other than anti-F1 (AOTF) in the recovered patients for finding new vaccine candidates and serodiagnostic markers, the protein microarray containing 218 known or putative virulence-associated proteins of *Y. pestis* was used to profile the antibody response in the recovered patients. According to the results of UPT-LF of the F1 antibody, the sera with similar F1 antibody values and those from the patients infected at the same years were pooled because of the limited quantity of each serum. Finally, 21 pooled sera were profiled for the antibody against the proteins on the microarray.

Figure 2 provides an overview of the antibody profiles according to the years of infection. The antibody responses to 20 proteins were found in at least one pooled sera of the patients infected in 2005, 1 year before the collection of sera. Four of them, YPO1089, YPO1435, YPMT1.62c, and YPMT1.86a, disappeared in all pooled sera of the patients infected after 2003.

In the patients who were recovered for more than 5 years, al-

TABLE 3 Antibody responses against F1 in the different gender and age groups of plague patients

Gender[a]	No. positive/tested (positive rate %) between age groups[b]		Total
	<18	≥18	
Female	6/7 (85.7)	17/24 (70.4)	23/31 (74.2)
Male	9/9 (100)	19/25 (76.0)	28/34 (82.4)
Total	15/16 (93.8)	36/49 (73.5)	51/65 (78.5)

[a] No difference was found between females and males ($P = 0.424$, x^2 test) in terms of F1 antibody-positive rate.
[b] No difference was found between the two age groups ($P = 0.087$, x^2 test).

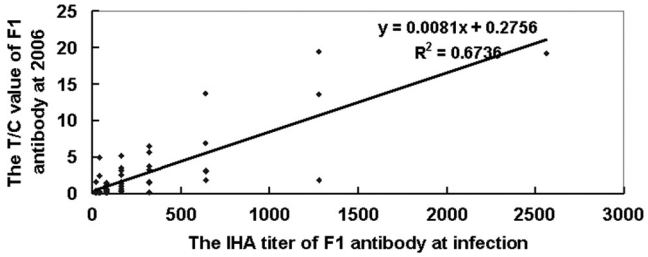

FIG 1 Correlations between the IHA titer at acute infection and the UPT value against F1 antigen of corresponding patients in 2006. T/C on the *y* axis represents the value of test/control for determining UPT results (positive or negative). The cutoff value is 0.1.

TABLE 4 The UPT results of the control people and EV76-immunized persons

People from areas of endemicity		People from areas of nonendemicity		EV76-immunized persons	
No.	The results of UPT in 2006 (T/C)	No.	The results of UPT in 2006 (T/C)	No.	The results of UPT in 2006 (T/C)
1	0.195	1	0.234	1	3.153
2	0.245	2	0.015	2	0.146
3	0.125	3	0.004	3	0.1
4	0.123	4	0.01	4	0.639
5	0.267	5	0.041	5	1.074
6	0.968	6	0.014	6	0.558
7	0.068	7	0.017	7	0.193
8	0.097	8	0.045	8	0.206
9	0.03	9	0.031	9	0.243
10	0.041	10	0.012	10	0.509
11	0.026	11	0.005	11	0.522
12	0.007	12	0.013	12	0.119
13	0.016	13	0.024	13	0.131
14	0.013	14	0.015	14	5.351
15	0.014	15	0.015	15	1.368
16	0.012	16	0.036	16	0.212
17	0.007	17	0.036	17	0.267
18	0.006	18	0.027	18	0.549
19	0.004	19	0.015	19	1.57
20	0.015	20	0.013	20	3.608
21	0.027	21	0.017	21	0.278
22	0.005	22	0.015	22	0.135
23	0.006	23	0.024	23	0.102
24	0.003	24	0.024	24	0.611
25	0.025	25	0.008	25	0.193
26	0.003	26	0.03	26	0.722
27	0.045	27	0.027	27	0.232
28	0.015	28	0.014	28	0.812
29	0.016	29	0.028	29	0.173
30	0.025	30	0.063	30	1.849
31	0.031	31	0.019	31	0.984
32	0.006	32	0.009	32	3.086
33	0.045	33	0.006	33	0.438
34	0.002	34	0.028	34	0.788
35	0.009	35	0.031	35	0.522
36	0.004	36	0.062	36	1.404
37	0.008	37	0.026	37	0.046
38	0.02	38	0.067	38	0.014
39	0.006	39	0.029	39	0.017
40	0.019	40	0.067	40	0.072
41	0.006	41	0.097	41	0.061
42	0.078	42	0.031	42	0.015
43	0.026	43	0.067	43	0.051
44	0.073			44	0.049
45	0.034			45	0.07
46	0.061			46	0.101
47	0.055			47	0.018
48	0.06			48	0.077
				49	0.038
				50	0.079

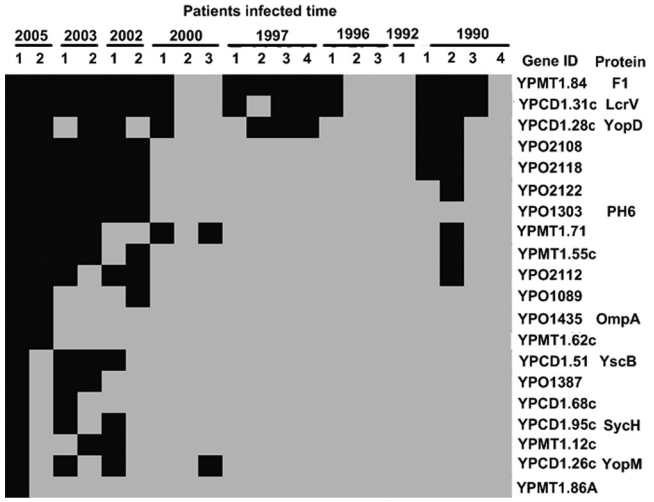

FIG 2 Antibody profiles for recovered plague patients determined by microarray analysis. The designation (number) of each pooled sera and the year of infection are indicated at the top, and the *Y. pestis* proteins which are designated with the CO92 gene definition are listed on the right side. Black represents positive reactivity whereas gray represents the absence of reactivity. The negative controls were not indicated in this figure because they were all negative.

most all AOTF antibodies disappeared, except for those to LcrV and YopD. The antibodies against LcrV and YopD were stable and were detected in most recovered patients (Fig. 2). Both proteins are encoded by type III secretion system and are essential for the virulence of *Y. pestis*. LcrV is the other vaccine target of plague, and the antibody against it prevents Yop-dependent growth of *Y. pestis* (22). Although there are numerous studies about longevity and variation of the F1 antibody in humans, the present study is the first to investigate the time of persistence of the antibody against LcrV. The long-term persistence of the LcrV antibody suggests that it is an important protective component against *Y. pestis* in humans. YopD is the other protein besides LcrV which provides partial protection of mice against nonencapsulated *Y. pestis* by subcutaneous challenge (2). However, the role of YopD in vaccine development has not been investigated further. The detection of its antibody in the recovered patients indicates its potential for serodiagnosis and protection against infection caused by F1-negative strains.

Memory cellular responses to F1 and LcrV in the recovered plague patients. Although the positive rate of the F1 antibody in patients was high, approximately 30% of the recovered patients remained seronegative to both F1 and LcrV. Moreover, the amount of antibodies in some patients was very low. To assess the cellular responses in plague patients, the T cell responses to recombinant F1 and LcrV antigens were studied utilizing 7 plague patients who recovered from plague 4 or 6 years ago, as well as 4 healthy controls from areas where plague is nonendemic. Although the cells of the patient that were stimulated with LcrV and F1 proteins produced IFN-γ, its level was not significantly different compared with those of the controls ($P > 0.05$) (Fig. 3).

Y. pestis is a facultatively intracellular bacterium during the early phase of infection. Cell-mediated immune responses should play an important role in the defense against this pathogen (21). Vaccination with live attenuated *Y. pestis* (KIM5 pCD1$^+$, pMT$^+$,

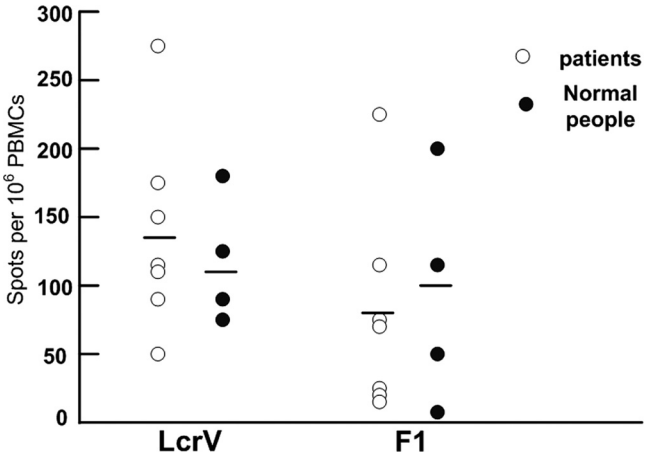

FIG 3 IFN-γ-producing cells in PBMCs from 7 patients and 4 healthy controls in response to F1 and LcrV proteins.

pPCP$^+$, pgm$^-$) primes CD4 and CD8 T cells that synergistically protect against lethal pulmonary *Y. pestis* infection (23). An earlier study considered that the effective treatment by anti-F1 antibodies in the mouse model is also required for T cells (11). At present, only a few data concerning the immunodominant target for the T cell response and the persistence of cell-mediated immunity in plague patients are available. In the present study, memory T cell responses against the F1 and LcrV proteins were not detected by ELISpot assay in plague patients who recovered from infection 4 or 6 years ago. Several explanations could be proposed to explain why the T cellular responses to F1 and LcrV are negative. First, as indicated by the results, F1 and LcrV are not the dominant T cell antigens. In the experiment on mice models immunized with EV76 vaccine strain, F1 antigens also failed to induce strong T cell responses (16). Second, the cellular immunities to F1 and LcrV antigens were produced in the acute and convalescence phases of infection but could not persist for a longer period of time. In order to confirm this possibility, the cellular responses to F1 and LcrV in patients infected 2 years ago were studied and blood samples were collected from the patients. Unfortunately, since the plague patients lived in remote small villages and the PBMCs could not be isolated within 24 h, the quality of the isolated cells could not meet the requirement of ELISpot assay. Evidence shows that cell-mediated immunity-related cytokines (interleukin-2 [IL-2] and IFN-γ) could not be detected in sera from primary pneumonic plague patients during infection. Only IL-6 could be detected in these patients (31). Nevertheless, based on our results and related literature, we speculate that F1 and LcrV may not be the dominant T cell antigens for the long-term defense against plague in humans. This provides a huge challenge to the plague vaccine development which is based only on F1 and LcrV antigens. There must be other proteins that play roles in stimulating cellular protective responses. A previous study conducted in our lab found that 34 proteins could stimulate strong T cell responses in EV76-immunized mice. Nine of the proteins provide partial protection against the challenge of a low dose of *Y. pestis*, independently. We also detected these 9 proteins for their ability to induce cellular immunity in plague patients (16). Unfortunately, none produced significantly different cell spots between patients and controls (data not shown).

Conclusions. In the present study, we analyzed the humoral and cellular immune responses to *Y. pestis* proteins F1 and LcrV in patients who were recovered from plague infection for several years to more than a decade to gain insight into the protective mechanism against plague. This is the first report on the study of both humoral and cellular responses against *Y. pestis* in humans. Antibody to F1 can persist in the recovered patients for more than 10 years, and antibodies to LcrV and YopD could also be present for a longer period of time. Specific memory T cell responses to F1 and LcrV could not be detected in plague patients 4 to 6 years postinfection. These results highlight the urgent need to develop effective live attenuated *Y. pestis* as a potential vaccine candidate (33).

ACKNOWLEDGMENTS

Financial support for this work came from the National Natural Science Foundation of China (grant 30930001), the key project of the Chinese Ministry of Education (grant 210144), and the young scholar project of the Department of Health, Hubei Province (grant QJX2010-24).

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